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(54) Title: HB-954 AS A TARGET FOR MODULATING ANGIOGENESIS

(57) Abstract: The invention describes assays for the identification of compounds useful for the modulation of angiogenesis. The methods of the invention involve cell-free and cell-based assays that identify compounds which bind to and/or activate or inhibit the activity of HB-954, a G protein- coupled receptor, optionally followed by an in vivo assay of the effect of the compound on angiogenesis. The invention also describes compounds which bind to and/or activate or inhibit the activity of HB-954 as well as pharmaceutical compositions comprising such compounds. In addition, the invention includes nucleic acid molecules comprising a nucleotide sequence encoding all or a portion of HB-954, gene therapy vectors comprising such sequences, polypeptides comprising all or a portion of HB-954 and antibodies directed against HB-954.

HB-954 AS A TARGET FOR MODULATING ANGIOGENESIS

BACKGROUND OF THE INVENTION

Angiogenesis, the development of new blood vessels from an existing vascular bed, is a complex multistep process that involves the degradation of components of the extracellular matrix and then the migration, proliferation and differentiation of endothelial cells to form tubules and eventually new vessels. Angiogenesis is important in normal physiological processes including, by example and not by way of limitation, embryo implantation; embryogenesis and development; and wound healing. Excessive angiogenesis is also involved in pathological conditions such as tumour cell growth and non-cancerous conditions such as neovascular glaucoma, rheumatoid arthritis, psoriasis and diabetic retinopathy. The vascular endothelium is normally quiescent. However, upon activation, endothelial cells proliferate and migrate to form a primitive tubular network which will ultimately form a capillary bed to supply blood to developing tissues including a growing tumour.

The G-protein-coupled receptors (GPCR) form an important class of peptide-binding receptors. The various members of the GPCR family mediate a wide variety of intercellular signals. Members of the GPCR family have seven helical domains which span the cell membrane and are linked by three extracellular loops and three intracellular loops.

SUMMARY OF THE INVENTION

The invention provides assays for the identification of compounds useful for the modulation of angiogenesis. Such compounds are useful for the treatment of angiogenesis related diseases. The methods of the invention involve cell-free and cell-based assays that identify compounds which bind to and/or activate or inhibit the activity of HB-954, a G protein-coupled receptor. The assays are optionally followed by an in vivo assay of the effect of the compound on angiogenesis and/or angiogenesis related diseases.

In addition, the invention provides nucleic acid molecules comprising a nucleotide sequence encoding all or a portion of HB-954, polypeptides comprising all or a portion of HB-954, antibodies directed against HB-954.

The invention also describes compounds which bind to and/or activate or inhibit the activity of HB-954 as well as pharmaceutical compositions comprising such compounds.

The invention also provides pharmaceutical compositions comprising a compound identified using the screening methods of the invention as a well as methods for preparing such compositions by combining such a compound and a pharmaceutically acceptable carrier. Also within the invention are pharmaceutical compositions comprising a compound identified using the screening assays of the invention packaged with instructions for use.

DETAILED DESCRIPTION OF THE INVENTION

Surprisingly if was found that a GPCR, named HB-954, GenBank Accession number D38449 (see Example 1), has an endothelial preferred pattern of expression, and that levels of its mRNA are induced by two distinct proangiogenic pathways, ie. that of sphingosine-1-phosphate SPP sphingosine-1-phosphate and VEGF (see Table 1).

HB-954 is homologous to the Orexin Receptor family of GPCRs which recogize neuropeptide ligands. Surprisingly, the findings of the present invention now link the endothelial-specific GPCR HB-954, and its putative protein ligand to the biology of endothelial cells, and to the process of angiogenesis.

Hata et al. (Biochimica et Biophysica Acta Vol 1261(1) March 14, 1995 pp121-125) have originally described the full-length cDNA clone HB-954, isolated from a human fetal brain library. The amino acid sequence of HB-954 deduced by Hata et al. contains four putative glycosylation sites in the N-terminal part, seven presumed transmembrane domains, and a large cytosolic domain in the C-terminal part.

Table 1: Relative Levels of endothelial-specific GPCR mRNA expression detected with the 1834_at probe set on the Affymetrix HG U95A chip*.

tissue sample	**relative level of HB-954 mRNA expression** (from 3 independent experiments)		
quiescent EC	89.5	35.9	10.2
proliferating EC	92.4	123.4	29.1
SPP treated EC	156.1	174.4	119.9
VEGF treated EC	171.5	140.6	126.9
SPP + VEGF treated EC	160.6	84.8	242.1; 324.4

* Proliferating HUVECs were in continuous culture, all other HUVEC samples were synchronized by overnight incubation in growth factor depleted conditions and then stimulation with one of Sphingosine 1 Phosphate, VEGF or both for 6 hours. Negative values were adjusted to zero. All other cells and tissues tested with the exception of angiopoietin treated smooth muscle cells did not show detectable levels of expression.

Screening Assays

The present invention provides methods for identifying compounds which can be used for the modulation of angiogenesis and for the treatment of a angiogenesis related diseases. The methods entail identifying candidate or test compounds which bind HB-954 and/or have a stimulatory or inhibitory effect on the activity or the expression of HB-954. Preferably, the identification of candidate or test compounds is followed by further determining which of the compounds that bind HB-954 or have a stimulatory or inhibitory effect on the activity or the expression of HB-954 have an effect on angiogenesis in an in vivo assay (effective compounds of the invention).

Candidate or test compounds or agents which bind HB-954 and/or have a stimulatory or inhibitory effect on the activity or the expression of HB-954 are identified in assays that employ either cells which express a form of HB-954 (cell-based assays) or isolated HB-954 (cell-free assays). The various assays of the invention can employ a variety of forms of HB-954, such as full-length HB-954, a biologically active fragment of HB-954, or a fusion protein which includes all or a portion of HB-954.

The assay can be a binding assay entailing direct or indirect measurement of the binding of a test compound or known HB-954 ligand to HB-954.

Thus, in one aspect of the invention there is provided a method for identifying a compound useful for modulating angiogenesis, the method comprising the steps of: a) contacting a test compound with a HB-954 polypeptide and b) determining whether the test compound binds to the HB-954 polypeptide.

Binding of the test compound to the HB-954 polypeptide can be determined either directly or indirectly as described above. In one embodiment, the assay includes contacting the HB-954 polypeptide with a known compound which binds the HB-954 polypeptide to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with the HB-954 polypeptide, wherein determining the ability of the test compound to interact with the HB-954 polypeptide comprises determining the ability of the test compound to preferentially bind to the HB-954 polypeptide as compared to the known compound.

Preferred is a method wherein the binding to the HB-954 polypeptide is within a K_D range of $10e^{-6}$ to $10e^{-13}$, preferably within a range of $10e^{-8}$ to $10e^{-12}$.

The assay can be in a competitive binding format.

Thus, in a further aspect of the invention there is provided a method for identifying a compound useful for modulating angiogenesis, the method comprising: a) contacting a HB-954 ligand with a HB-954 polypeptide in the presence and absence of a test compound and b) determining whether the test compound alters the binding of the HB-954 ligand to the HB-954 polypeptide.

The assay can also be an activity assay, such as a cellular activity assay, entailing direct or indirect measurement of the activity of HB-954.

Thus, in another aspect of the invention there is provided a method for identifying a compound useful for modulating angiogenesis, the method comprising: a) contacting a test compound with a cell expressing a HB-954 polypeptide and b) determining whether the test compound alters activity of the HB-954 polypeptide in said cell.

Determining the ability of the test compound to modulate the activity of the membrane-bound form of HB-954 can be accomplished by any method suitable for measuring the activity of HB-954, e.g., any method suitable for measuring the activity of a G- protein coupled receptor or other seven-transmembrane receptor.

The activity of a seven-transmembrane receptor can be measured in a number of ways, not all of which are suitable for any given receptor. Among the measures of activity are: alteration in intracellular Ca^{2+} concentration, activation of phospholipase C, alteration in intracellular inositol triphosphate (IP_3) concentration, alteration in intracellular diacylglycerol (DAG) concentration, and alteration in intracellular adenosine cyclic 3', 5'-monophosphate (cAMP) concentration.

It can also be accomplished, for example, by determining the ability of HB-954 to bind to or interact with a target molecule. The target molecule can be a molecule with which HB-954 binds or interacts with in nature, for example, a molecule on the surface of a cell which expresses HB-954, a molecule on the surface of a second cell, a molecule in the extracellular milieu, a molecule associated with the internal surface of a cell membrane or a cytoplasmic molecule. The target molecule can be a component of a signal transduction pathway which facilitates transduction of an extracellular signal (e.g., a signal generated by binding of a HB-954 ligand to HB-954) through the cell membrane and into the cell. The target molecule can be, for example, a second intracellular protein which has catalytic activity or a protein which facilitates the association of downstream signaling molecules with HB-954. A HB-954 ligand is one example of a HB-954 target molecule.

The screening assays of the invention may be combined with an in vitro or vivo assay entailing measuring the effect of the test compound on angiogenesis or angiogenesis related diseases.

Thus, the above methods of the invention may further comprise the steps of: c) adding a compound identified by a method of the invention to an assay for modulation of

angiogenesis; d) determining whether the compound modulates angiogenesis; and e) identifying a compound that modulates angiogenesis as a compound useful for the treatment of angiogenesis related diseases.

As described in greater detail below, the test compound can be obtained by any suitable means, e.g., from conventional compound libraries. In one embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of a membrane-bound form of HB-954. Determining the ability of the test compound to bind to a membrane-bound form of HB-954 can be accomplished, for example, by coupling the test compound with a radioisotope or enzymatic label such that binding of the test compound to the HB-954-expressing cell can be measured by detecting the labeled compound in a complex.

In various embodiments of the above assay methods of the present invention, it may be desirable to immobilize the HB-954 polypeptide (or a HB-954 target molecule) to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to the HB-954 polypeptide, or interaction of the HB-954 polypeptide with a target molecule in the presence and absence of a candidate compound, can be accomplished by methods well known in the art.

The screening assay can also involve monitoring the expression of HB-954. For example, modulators of expression of HB-954 can be identified in a method in which a cell is contacted with a candidate compound and the expression of HB-954 protein or mRNA in the cell is determined. The level of expression of HB-954 protein or mRNA the presence of the candidate compound is compared to the level of expression of HB-954 protein or mRNA in the absence of the candidate compound. The candidate compound can then be identified as a modulator of expression of HB-954 based on this comparison. For example, when expression of HB-954 protein or mRNA protein is statistically significantly greater in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of HB-954 protein or mRNA expression. Alternatively, when expression of HB-954 protein or mRNA is statistically significantly less in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of HB-954 protein or

mRNA expression. The level of HB-954 protein or mRNA expression in the cells can be determined by methods described below.

Angiogenesis related diseases and angiogenesis models

"Angiogenesis related diseases" within the meaning of the invention include but are not limited to coronary artery disease, peripheral vascular disease, wound healing, islet cell transplantation, fracture and tendon repair , reconstructive surgery, tissue engineering, restenosis, cancer, age-related macular degeneration, diabetic retinopathy, rheumatoid arthritis, psoriasis, obesity, hemangioma/AIDS-related kaposi's sarcoma, atherosclerotic plaque rupture.

In a preferred embodiment effective compounds identified with the assays of the invention further described herein primarily inhibit the growth of blood vessels and are thus, for example, effective against a number of diseases associated with deregulated angiogenesis, especially diseases caused by ocular neovascularisation, especially retinopathies, such as diabetic retinopathy or age-related macular degeneration, psoriasis, haemangioblastoma, such as haemangioma, mesangial cell proliferative disorders, such as chronic or acute renal diseases, e.g. diabetic nephropathy, malignant nephrosclerosis, thrombotic microangiopathy syndromes or transplant rejection, or especially inflammatory renal disease, such as glomerulonephritis, especially mesangio-proliferative glomerulonephritis, haemolytic-uraemic syndrome, diabetic nephropathy, hypertensive nephrosclerosis, atheroma, arterial restenosis, autoimmune diseases, acute inflammation, fibrotic disorders (e.g. hepatic cirrhosis), diabetes, neurodegenerative disorders and especially neoplastic diseases (solid tumours, but also leukemias and other "liquid tumours", especially those expressing c-kit, KDR or flt-1), such as especially breast cancer, cancer of the colon, lung cancer (especially small-cell lung cancer), cancer of the prostate or Kaposi's sarcoma. An effective compound of the invention may inhibit the growth of tumours and is especially suited to preventing the metastatic spread of tumours and the growth of micrometastases.

The angiogenesis modulating activity of the compound can be tested in vitro by a variety of methods such as endothelial cell migration, proliferation, apoptosis, and tube formation. Additionally, more complex ex vivo (Nicosia R.F. and Ottinetti, A. Laboratory Investigation, 63, p115-122, 1990) and in vivo models can be used to assess the activity

of angiogenesis modulating compounds (reviewed in Nat Med 1997 Nov;3(11):1203-8). Common models for this include:

1. The matrigel angiogenesis model in which angiogenic : Ancellin N. et al., J. Biol. Chem. 277, 6667-6675, 2002.

2. The corneal pocket assay: Gimbrone, M.A.J., et al., J. Natl. Cancer Inst. 52, 413-427, 1974

3. The chick embryonic chorioallantoic membrane assay: Nguyen, M., et al., Microvascular Research, 47, 31-40, 1994.

The efficacy of the compounds of the invention as it relates to coronary artery disease and peripheral vascular diseases can be modeled as follows. The most commonly used coronary disease models is an ameroid constriction model (Lamping, KA at el., J. Pharmacol Exp. Ther 229, 359-363, 1984). A second model that may mimic the human condition more accurately is a repetitive occlusion model (Kersten JR et al., American J. Physiol. 268, H720-728, 1995). Rabbit, rat, and mouse have been used to model peripheral vascular diseases (Hershey JC et al., Cardiovascular Research 49, 618-625, 2001 and Mack CA et., J. Vascular Surgery, 27, 699-709, 1998).

The efficacy of the compound s of the invention as it relates to age-related macular degeneration or to diabetic retinopathy can be demonstrated in vivo as follows:

In vivo inhibition of choroidal neovascularization is modeled by a laser photocoagulation to rupture Bruch's membrane (Mori et al., American J. of Pathology, 159, 313-320, 2001). Ischemic retinopathy is modeled by first placing neonatal mouse in an hyperoxia environment with subsequent return to normal oxygen tension (Smith LEH et al., Invest. Ophthalmol. Vis. Sci. 35, 101-111, 1994).

The antitumor efficacy of the compounds of the invention can be demonstrated in vivo as follows: In vivo activity in the nude mouse xenotransplant model: female BALB/c nude mice (8–12 weeks old), Novartis Animal Farm, Sisseln, Switzerland) are kept under sterile conditions with water and feed ad libitum. Tumors are induced either by subcutaneous injection of tumor cells into mice (for example, Du 145 prostate carcinoma cell line (ATCC No. HTB 81; see Cancer Research 37, 4049-58 (1978)) or by implanting tumor fragments (about 25 mg) subcutaneously into the left flank of mice using a 13-gauge trocar needle under Forene® anaesthesia (Abbott, Switzerland). Treatment with the test compound is started as soon as the tumor has reached a mean volume of 100 mm^3. Tumor growth is measured two to three times a week and 24 hours after the last treatment by determining the length of two perpendicular axes. The tumor volumes are calculated in accordance with published methods (see Evans et al., Brit. J. Cancer 45, 466-8 [1982]). The antitumor efficacy is determined as the mean increase in tumor volume of the treated animals divided by the mean increase in tumor volume of the untreated animals (controls) and, after multiplication by 100, is expressed as T/C%. Tumor regression (given in %) is reported as the smallest mean tumor volume in relation to the mean tumor volume at the start of treatment. The test compound is administered daily by gavage.

As an alternative other cell lines may also be used in the same manner, for example:
- the MCF-7 breast adenocarcinoma cell line (ATCC No. HTB 22; see also J. Natl. Cancer Inst. (Bethesda) 51, 1409-16 [1973]);
- the MDA-MB 468 breast adenocarcinoma cell line (ATCC No. HTB 132; see also In Vitro 14, 911-15 [1978]);
- the MDA-MB 231 breast adenocarcinoma cell line (ATCC No. HTB 26; see also J. Natl. Cancer Inst. (Bethesda) 53, 661-74 [1974]);
- the Colo 205 colon carcinoma cell line (ATCC No. CCL 222; see also Cancer Res. 38, 1345-55 [1978]);
- the HCT 116 colon carcinoma cell line (ATCC No. CCL 247; see also Cancer Res. 41, 1751-6 [1981]);
- the DU145 prostate carcinoma cell line DU 145 (ATCC No. HTB 81; see also Cancer Res. 37, 4049-58 [1978]); and

- the PC-3 prostate carcinoma cell line PC-3 (ATCC No. CRL 1435; see also Cancer Res. 40, 524-34 [1980]).

The usefulness of a compound identified by the present invention in the treatment of arthritis as an example of an inflammatory rheumatic or rheumatoid disease can be demonstrated as follows:

The well-known rat adjuvant arthritis model (Pearson, Proc. Soc. Exp. Biol. 91, 95-101 (1956)) is used to test the anti-arthritic activity of compounds of the invention, or salts thereof. Adjuvant Arthritis can be treated using two different dosing schedules: either (i) starting time of immunisation with adjuvant (prophylactic dosing); or from day 15 when the arthritic response is already established (therapeutic dosing). Preferably a therapeutic dosing schedule is used. For comparison, a cyclooxygenase-2 inhibitor, such as 5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]thiophene or diclofenac, is administered in a separate group.

In detail, male Wistar rats (5 animals per group, weighing epproximately 200 g, supplied by Iffa Credo, France) are injected i.d. (intra-dermally) at the base of the tail with 0.1 ml of mineral oil containing 0.6 mg of lyophilised heat-killed Mycobacterium tuberculosis. The rats are treated with the test compound (3, 10 or 30 mg/kg p.o. once per day), or vehicle (water) from day 15 to day 22 (therapeutic dosing schedule). At the end of the experiment, the swelling of the tarsal joints is measured by means of a mico-calliper. Percentage inhibition of paw swelling is calculated by reference to vehicle treated arthritic animals (0 % inhibition) and vehicle treated normal animals (100 % inhibition).

On the basis of these studies, a compound identified by the present invention is appropriate for the treatment of inflammatory (especially rheumatic or rheumatoid) diseases.
In addition, there exist a number of transgenic models that are useful for angiogenesis and disease-relevant analyses e.g. cancer and cardiovascular diseases (reviewed in Hanahan D. et al., European J. Cancer 32A, 2386-2393, 1996 Carmeliet, P. and Collen, D., J. of Pathology, 190, 387-405, 2000).

Test Compounds

Suitable test compounds for use in the screening assays of the invention can be obtained from any suitable source, e.g., conventional compound libraries. The test compounds can also be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection.

Modeling of compounds

Computer modeling and searching technologies permit identification of compounds, or the improvement of already identified compounds, that can modulate HB-954 expression or activity. Having identified such a compound or composition, the active sites or regions are identified. Such active sites might typically be ligand binding sites. The active site can be identified using methods known in the art including, for example, from the amino acid sequences of peptides, from the nucleotide sequences of nucleic acids, or from study of complexes of the relevant compound or composition with its natural ligand. In the latter case, chemical or X- ray crystallographic methods can be used to find the active site by finding where on the factor the complexed ligand is found.

Next, the three dimensional geometric structure of the active site is determined. This can be done by known methods, including X-ray crystallography, which can determine a complete molecular structure. On the other hand, solid or liquid phase NMR can be used to determine certain intramolecular distances. Any other experimental method of structure determination can be used to obtain partial or complete geometric structures. The geometric structures may be measured with a complexed ligand, natural or artificial, which may increase the accuracy of the active site structure determined.

Finally, having determined the structure of the active site, either experimentally, by modeling, or by a combination, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. Such a search seeks compounds having structures that match the determined

active site structure and that interact with the groups defining the active site. Such a search can be manual, but is preferably computer assisted. These compounds found from this search are potential HB-954 modulating compounds.

Isolated Nucleic Acid Molecules

One aspect of the invention pertains to isolated nucleic acid molecules that encode HB-954 protein and fragments thereof.

A nucleic acid molecule of the present invention, e.g., a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:2, can be isolated using standard molecular biology techniques and the sequence information provided herein. Using all or a portion of the nucleic acid sequences of SEQ ID NO:2 as a hybridization probe, nucleic acid molecules of the invention can be isolated using standard hybridization and cloning techniques.

A nucleic acid molecule of the invention can comprise only a portion of a nucleic acid sequence encoding HB-954, for example, a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of HB-954. The nucleotide sequence determined from the cloning one gene allows for the generation of probes and primers designed for use in identifying and/or cloning allelic variants and other variants of HB-954. The probe/primer typically comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably about 50, 75, 100, 125, 150, 175, 200, 250, 300, 350 or 400 consecutive nucleotides of the sense or antisense sequence of SEQ ID NO: 2 of a naturally occurring mutant or allelic variant of SEQ NO: 2.

A nucleic acid fragment encoding a "biologically active portion" of HB-954 can be prepared by isolating a portion of SEQ ID NO:2 which encodes a polypeptide having a biological activity, expressing the encoded portion of the polypeptide protein and assessing the activity of the encoded portion of the polypeptide.

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence of SEQ ID NO: 2 due to degeneracy of the genetic code and thus encode the same protein as that encoded by the nucleotide sequence shown in SEQ ID NO:2.

In addition to naturally-occurring allelic variants of HB-954, the skilled person will further appreciate that changes can be introduced by mutation thereby leading to changes in the amino acid sequence of the encoded protein, without altering the biological activity of the protein. For example, one can make nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are not conserved or only semi-conserved among homologues of various species may be non-essential for activity and thus would be likely targets for alteration. Alternatively, amino acid residues that are conserved among the homologues of various species may be essential for activity and thus would not be likely targets for alteration.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding HB-954 that contain changes in amino acid residues that are not essential for activity. Such polypeptides differ in amino acid sequence from SEQ ID NO:1 yet retain biological activity. In one embodiment, the isolated nucleic acid molecule includes a nucleotide sequence encoding a protein that includes an amino acid sequence that is at least about 85%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:1.

In one embodiment, a mutant polypeptide that is a variant of HB-954 can be assayed for: (1) the ability to form protein:protein interactions with proteins in a signaling pathway of HB-954; (2) the ability to bind a ligand of HB-954; or (3) the ability to bind to an intracellular target protein of HB-954. In another embodiment, the mutant polypeptide can be assayed for the ability to modulate angiogenesis.

Antisense nucleic acid molecules

The present invention encompasses antisense nucleic acid molecules, i.e., molecules which are complementary to a sense nucleic acid encoding HB-954 or to a fragment thereof, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be

complementary to an entire coding strand, or to only a portion thereof, e.g., all or part of the protein coding region (or open reading frame). An antisense nucleic acid molecule can be antisense to all or part of a noncoding region of the coding strand of a nucleotide sequence encoding HB-954. The noncoding regions ("5' and 3' untranslated regions") are the 5' and 3' sequences which flank the coding region and are not translated into amino acids.

An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated in situ such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding HB-954 to thereby inhibit expression, e.g., by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix.

The invention also encompasses ribozymes. Ribozymes are generated by methods well known in the art. They are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region.

The invention also encompasses nucleic acid molecules which form triple helical structures. For example, expression of HB-954 can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the gene encoding the polypeptide (e.g., the promoter and/or enhancer) to form triple helical structures that prevent transcription of the gene in target cells.

In certain embodiments, the nucleic acid molecules of the invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids. As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, e.g., DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as are described in the art.

Vectors and gene therapy

The polynucleotides of the invention encoding HB-954 protein or the antisense polynucleotide which are complementary to at least a portion of a polynucleotide encoding HB-954 protein may be contained within an appropriate expression vehicle which may be transduced into a cell. Such expression vehicles include, but are not limited to, plasmids, eukaryotic vectors, prokaryotic vectors (such as, for example, bacterial vectors), and viral vectors, which may be used in gene therapy applications.

In one embodiment, the vector is a viral vector. Viral vectors which may be employed include RNA virus vectors (such as retroviral vectors or lentiviral vectors), and DNA virus vectors (such as adenoviral vectors, adeno-associated virus vectors, herpes virus vectors, and vaccinia virus vectors). When an RNA virus vector is employed, in constructing the vector, the polynucleotide encoding the antisense polynucleotide is in the form of RNA. When a DNA virus vector is employed, in constructing the vector, the polynucleotide encoding the antisense polynucleotide is in the form of DNA.

In one embodiment, the viral vector is a retroviral vector. Examples of retroviral vectors which may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus. The vector is generally a replication incompetent retrovirus particle. Preferred viral vectors include lentiviral

vectors, such as HIV or BIV based vectors in particular. Retro- and lentiviral vectors are useful as agents to mediate retroviral-mediated gene transfer into eukaryotic cells. Retro- and lentiviral vectors are generally constructed such that the majority of sequences coding for the structural genes of the virus are deleted and replaced by the gene(s) of interest. Most often, the structural genes (i.e., gag, pol, and env), are removed from the retroviral backbone using genetic engineering techniques known in the art.

The polynucleotides of the invention may be incorporated into the proviral backbone in several general ways. The most straightforward constructions are ones in which the structural genes of the retrovirus are replaced by a single gene which then is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Retroviral vectors may also be constructed which can introduce more than one gene into target cells. Usually, in such vectors one gene is under the regulatory control of the viral LTR, while the second gene is expressed either off a spliced message or is under the regulation of its own, internal promoter. Alternatively, two genes may be expressed from a single promoter by the use of an Internal Ribosome Entry Site.

Efforts are directed at minimizing the viral component of the viral backbone, largely to reduce the chance for recombination between the vector and the packaging-defective helper virus within packaging cells. A packaging-defective helper virus is necessary to provide the structural genes of a retrovirus, which have been deleted from the vector itself.

Isolated Proteins and Antibodies

One aspect of the invention pertains to isolated proteins, and biologically active portions thereof, as well as polypeptide fragments suitable for use as immunogen to raise antibodies directed against HB-954. In one embodiment, native HB-954 can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, polypeptides of the invention are produced by recombinant DNA techniques. Alternative to recombinant expression, HB-954 can be synthesized chemically using standard peptide synthesis techniques. This section describes HB-954 polypeptides, antibodies directed against HB-954, and

methods for making and using such polypeptides and antibodies. However, the same techniques can be employed to make and use human HB-954 polypeptides and anti-human HB-954 antibodies.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of heterologous protein (also referred to herein as a "contaminating protein"). When the protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, 10%, or 5% of the volume of the protein preparation. When the protein is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, i.e., it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. Accordingly such preparations of the protein have less than about 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or non-HB-954 chemicals.

Biologically active portions of HB-954 include polypeptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the protein, e.g., the amino acid sequence shown in SEQ ID NO:1, which include fewer amino acids than the full length protein, and exhibit at least one activity of the corresponding full-length protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the corresponding protein. A biologically active portion of a protein of the invention can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length. Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of the native form of HB-954.

Among the useful polypeptides are those having the amino acid sequence of SEQ ID NO:1. Other useful proteins are substantially identical (e.g., at least about 96%, 97%, 98%, 99%, or 99.5%) to any SEQ ID NO:1 and retain the functional activity of the protein of the corresponding naturally-occurring protein yet differ in amino acid sequence due to natural allelic variation or mutagenesis.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences. Preferably, the two sequences are the same length.

The determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403410. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

The invention also provides chimeric or fusion proteins. As used herein, a "chimeric protein " or "fusion protein" comprises all or part (e.g., a biologically active fragment) of HB-954 operably linked to a heterologous polypeptide (i.e., a polypeptide other than the same polypeptide of the invention). Within the fusion protein, the term "operably linked" is intended to indicate that the polypeptide of the invention and the heterologous

polypeptide are fused in-frame to each other. The heterologous polypeptide can be fused to the N-terminus or C- terminus of the HB-954 polypeptide.

Useful fusion proteins include fusions to FLAG®, a portion lacz, GST, calmodulin-binding peptide, Hiss, or HA. Vectors for preparing such fusions proteins are available from Clontech, Inc. (Palo Alto, Calif.) and Stratagene, Inc. (La Jolla, Calif.). In another embodiment, the fusion protein may contain a heterologous signal sequence at its N-terminus.

In yet another embodiment, the fusion protein is an immunoglobulin fusion protein in which all or part of HB-954 is fused to sequences derived from a member of the immunoglobulin protein family.

Chimeric and fusion protein of the invention can be produced by standard recombinant DNA techniques. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers.

The present invention also pertains to variants of HB-954. Such variants have an altered amino acid sequence which can function as either agonists or as antagonists. Variants can be generated by mutagenesis, e.g., discrete point mutation or truncation. An agonist can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of the protein. An antagonist of a protein can inhibit one or more of the activities of the naturally occurring form of the protein by, for example, competitively binding to a downstream or upstream member of a cellular signaling cascade which includes the protein of interest. Thus, specific biological effects can be elicited by treatment with a variant of limited function. Treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein can have fewer side effects in a subject relative to treatment with the naturally occurring form of the protein.

Variants of a protein of the invention which function as either agonists (mimetics) or as antagonists can be identified by screening combinatorial libraries of mutants, e.g., truncation mutants, of the protein of the invention for agonist or antagonist activity. In one embodiment, a variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of variants can be produced by, for example, enzymatically ligating a

mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential protein sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display). There are a variety of methods which can be used to produce libraries of potential variants of the polyepeptides of the invention from a degenerate oligonucleotide sequence.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected.

Another aspect of the invention pertains to antibodies directed against HB-954. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds an antigen, such as HB-954. A molecule which specifically binds to a given polypeptide of the invention is a molecule which binds the polypeptide, but does not substantially bind other molecules in a sample, e.g., a biological sample, which naturally contains the polypeptide. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope.

Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods for modulating angiogenesis.

In one aspect, the invention provides a method for modulating angiogenesis by administering a compound which modulates an activity of HB-954. Such methods are

useful for modulating angiogenesis both in patients having aberrant expression or activity of HB-954 or other patients which would benefit from administration of a compound which modulates activity of HB-954. Depending on the needs of the patient a HB-954 agonist or antagonist can be used for treating the subject.

The modulatory method of the invention involves contacting a cell with a compound that modulates one or more of the activities of HB-954. A compound that modulates activity can be a compound as described herein, such as a nucleic acid or a protein, a naturally-occurring cognate ligand of the polypeptide, a peptide, a peptidomimetic, or other small molecule. In one embodiment, the compound stimulates one or more of the biological activities of HB-954. Inhibitory compounds include antisense nucleic acid molecules and antibodies. Stimulation of activity or expression is desirable in situations in which activity or expression is abnormally low downregulated and/or in which increased activity is likely to have a beneficial effect. Conversely, inhibition of activity or expression is desirable in situations in which activity or expression is abnormally high or upregulated and/or in which decreased activity is likely to have a beneficial effect.

These modulatory methods can be performed in vitro (e.g., by culturing the cell with the compound) or, alternatively, in vivo (e.g, by administering the compound to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by unwanted expression or activity of HB-954 or a protein in the HB-954 signaling pathway. In one embodiment, the method involves administering a compound, e. g., a compound identified by a screening assay described herein; or combination of compounds that modulates, e.g., upregulates or downregulates expression or activity of HB-954 or a protein in the HB-954 signalling pathway. In another embodiment, the method involves administering a modulator of HB-954 as therapy to compensate for reduced or undesirably low expression or activity of HB-954 or a protein in the HB-954 signalling pathway.

Pharmaceutical Compositions

This invention further pertains to novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

The nucleic acid molecules, polypeptides, and antibodies of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such

compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Supplementary active compounds can also be incorporated into the compositions.

The invention includes pharmaceutical compositions comprising a modulator of HB-954 expression or activity as a well as methods for preparing such compositions by combining one or more such modulators and a pharmaceutically acceptable carrier. Also within the invention are pharmaceutical compositions comprising a modulator identified using the screening assays of the invention packaged with instructions for use, ie. for use of the pharmaceutical composition for treatment of angiogenesis related diseases.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral, transdermal, transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL®(BASF; Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy

syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from a pressurized container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Pat. No. 5,328,470) or by stereotactic injection (see, e.g., Chen et al. (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which

the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. For pharmaceutical compositions which include an antagonist of HB-954 activity, a compound which reduces expression of HB-954, or a compound which reduces expression or activity of a protein in the HB-954 signaling pathway (or some combination thereof), the instructions for administration will specify use of the composition for modulating angiogenesis.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLES

Example 1: Sequence listing

SEQ ID NO:1 - Amino acid sequence of HB-954, GenBank Accession number D38449:

MGHNGSWISPNASEPHNASGAEAAGVNRSALGEFGEAQLYRQFT
TTVQVVIFIGSLLGNFMVLWSTCRTTVFKSVTNRFIKNLACSGICASLVCVPFDIILS
TSPHCCWWIYTMLFCKVVKFLHKVFCSVTILSFPAIALDRYYSVLYPLERKISDAKSR
ELVMYIWAHAVVASVPVFAVTNVADIYATSTCTEVWSNSLGHLVYVLVYNITTVIVPV
VVVFLFLILIRRALSASQKKKVIIAALRTPQNTISIPYASQREAELHATLLSMVMVFI
LCSVPYATLVVYQTVLNVPDTSVFLLLTAVWLPKVSLLANPVLFLTVNKSVRKCLIGT
LVQLHHRYSRRNVVSTGSGMAEASLEPSIRSGSQLLEMFHIGQQQIFKPTEDEEESEA
KYIGSADFQAKEIFSTCLEGEQGPQFAPSAPPLSTVDSVSQVAPAAPVEPETFPDKYS
LQFGFGPFELPPQWLSETRNSKKRLLPPLGNTPEELIQTKVPKVGRVERKMSRNNKVS
IFPKVDS

SEQ ID NO:2 - Nucleic acid sequence of HB-954, GenBank Accession number D38449:

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1    tgggggcgtc ctccttcgtc cccgcccggc tgtcaagctg tgttctagcg gccgagggac
61   cgaggggggc taagaaaggg ggcgcccagc catgcagagg caaaaaggcg ctgcggaacg
121  gggtccccgt cgccagtgct gaggcaggag gtcggagcca caagtgaggg gctgggaagc
181  aggacccagc acgggcgtct tggcaggcgg ccgggcgcag ggccaggctg ctggggacgc
241  tcagggcttt ccacccaagc catgggcgct gtcgggcact cgggggtccc ctcgtggctc
301  cggccactcg gcgtgggcat tacgttggct tcacatcgcc atccagcctc gaagccaaca
361  ggactgaaaa atagcttcgg ccaaacgttc tcctcccgct aaggagaggg gtcgagtgcg
421  tcagcccgag gggactggag agggatgccc tagccctcga ggggcggagg acccgcggtt
481  gaaggaggca gcgggagcgg agagcgccct ccttgaccat cgaatgcctc cttctgtgtt
541  tccattcctg tcgagtgggc tgggccacgc tgaccaccct ggaggaggga cggacgacgc
601  tcggcgggct ctgaccgtgc cgccttcttg tggctgctga ctgggatcca ggagggagtg
661  ggcatggggc gcagccgcgc ctccctccct ccccgcctcc cgggcgccgg ggttggcgat
721  gtggagacgt gaggggaccc gtcggctgct ccggcttctc caggactccg ccaggcgccc
781  gcgcgtccct cctcacccgg aggaggagag gctccgcgcg gggctccgag gcgggcggcg
841  cgcggagccg gagtcccagc ctcgccatgg gacataacgg gagctggatc tctccaaatg
901  ccagcgagcc gcacaacgcg tccggcgccg aggctgcggg tgtgaaccgc agcgcgctcg
961  gggagttcgg cgaggcgcag ctgtaccgcc agttcaccac caccgtgcag gtcgtcatct
1021 tcataggctc gctgctcgga aacttcatgg tgttatggtc aacttgccgc acaaccgtgt
1081 tcaaatctgt caccaacagg ttcattaaaa acctggcctg ctcggggatt tgtgccagcc
1141 tggtctgtgt gcccttcgac atcatcctca gcaccagtcc tcactgttgc tggtggatct
1201 acaccatgct cttctgcaag gtcgtcaaat ttttgcacaa agtattctgc tctgtgacca
1261 tcctcagctt ccctgctatt gctttggaca ggtactactc agtcctctat ccactggaga
1321 ggaaaatatc tgatgccaag tcccgtgaac tggtgatgta catctgggcc catgcagtgg
1381 tggccagtgt ccctgtgttt gcagtaacca atgtggctga catctatgcc acgtccacct
1441 gcacggaagt ctggagcaac tccttgggcc acctggtgta cgttctggtg tataacatca
1501 ccacggtcat tgtgcctgtg gtggtggtgt tcctcttctt gatactgatc cgacgggccc
1561 tgagtgccag ccagaagaag aaggtcatca tagcagcgct ccggacccca cagaacacca
1621 tctctattcc ctatgcctcc cagcgggagg ccgagctgca cgccaccctg ctctccatgg
1681 tgatggtctt catcttgtgt agcgtgccct atgccaccct ggtcgtctac cagactgtgc
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1741 tcaatgtccc tgacacttcc gtcttcttgc tgctcactgc tgtttggctg cccaaagtct
1801 ccctgctggc aaaccctgtt ctctttctta ctgtgaacaa atctgtccgc aagtgcttga
1861 tagggaccct ggtgcaacta caccaccggt acagtcgccg taatgtggtc agtacaggga
1921 gtggcatggc tgaggccagc ctggaaccca gcatacgctc gggtagccag ctcctggaga
1981 tgttccacat tgggcagcag cagatcttta agcccacaga ggatgaggaa gagagtgagg
2041 ccaagtacat tggctcagct gacttccagg ccaaggagat atttagcacc tgcctggagg
2101 gagagcaggg gccacagttt gcgccctctg ccccacccct gagcacagtg gactctgtat
2161 cccaggtggc accggcagcc cctgtggaac ctgaaacatt ccctgataag tattccctgc
2221 agtttggctt tgggcctttt gagttgcctc ctcagtggct ctcagagacc cgaaacagca
2281 agaagcggct gcttcccccc ttgggcaaca ccccagaaga gctgatccag acaaaggtgc
2341 ccaaggtagg cagggtggag cggaagatga gcagaaacaa taaagtgagc atttttccaa
2401 aggtggattc ctagcaagga ttgtaaattc ttggaagcaa cgggggggctt ccatattccc
2461 accagagtgt gggaatgctg tggccatgtg attgtatgat ctccttgcaa ctcagtgtga
2521 gttgattcct ccaatatggg ccagatgctt ttgaatgata gggaaatcta cataaaatcc
2581 agtgtcctct ttattgaggg agtatatgta tccatctcag tgatccatgt ccttagtgaa
2641 gtccacatta ttctctgtgg ggacaagagc tgggcagttt tgaatgggtc ttgaggtggg
2701 taccccatgt gcactttctg aggatgcctc acttccctgg gctctgcaga gaacacacag
2761 agagaagact ttcagagctc acaggagcag ggagcaggag cactctaagg gaattc
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Example 2

GENE CHIP ANALYSIS OF mRNA EXPRESSION

Human Umbilical Vein Endothelial Cells (HUVEC) were grown in Endothelial Cell Growth Media (EGM) (Clonetics Inc., Palo Alto, CA) containing 2% FBS. Proliferating HUVEC were grown in EGM and harvested at a subconfluent stage, 24 hr after media addition. For the other treatment groups, HUVECs (50% to 70% confluent) were incubated with Endothelial Cell Basal Medium (EBM) + 2% charcoal-stripped FBS (CFBS) for 24 hours to render the cells quiescent. After 24 hours in EBM + 2% CFBS, the media was removed and one of the following mixtures was added: EBM + 0.1% CFBS, EBM + 0.1% CFBS + 1μM sphingosine-1-phosphate (SPP), EBM + 0.1% CFBS + 0.05 μg/ml VEGF, or EBM + 0.1% CFBS + 1μM SPP + 0.05 μg/ml VEGF. Six hours after the addition of the treatment conditions, total RNA was extracted using TRIzol Reagent via the protocol recommended by the vendor (Invitrogen, Carlsbad, CA). Total RNA was additionally purified via affinity column using the RNeasy Purification Kit (Qiagen, Valencia CA). RNA from three independent sets were used for RNA expression profiling. The total RNA was used for first and second strand cDNA synthesis by reverse transcriptase (Life Technologies). The cDNA was subsequently used to generate biotin-labeled cRNA probe using ENZO BioArray High Yield RNA transcript labeling kit. The cRNA wa purified by RNeasy Purification Kit (Qiagen) and quality verified by agarose gel analysis. The cRNA was fragmented prior to hybridization to the Affymetrix HG U95A array and hybridization was performed according to the Affymetrix protocol. The data was

acquired via scanning with the Gene Array scanner (Affymetrix) and the results analyzed by Novartis proprietary software suite for graphic representation and rapid complex integrated analysis of microarray expression data across multiple experiments.

Claims

1. A method for identifying a compound useful for modulating angiogenesis, the method comprising the steps of: a) contacting a test compound with a HB-954 polypeptide; and b) determining whether the test compound binds to the HB-954 polypeptide.

2. The method of claim 1, wherein the binding to the HB-954 polypeptide is within a K_D range of $10e^{-6}$ to $10e^{-13}$, preferably within a range of $10e^{-8}$ to $10e^{-12}$.

3. The method of claim 1 or 2, said method further comprising the steps of: c) adding a compound identified as binding to the HB-954 polypeptide in step (b) to an assay for the modulation of angiogenesis; d) determining whether the compound modulates angiogenesis; and e) identifying a compound that modulates angiogenesis in step (d) as a compound useful for the treatment of angiogenesis related diseases.

4. A method for identifying a compound useful for modulating angiogenesis, the method comprising: a) contacting a HB-954 ligand with a HB-954 polypeptide in the presence and absence of a test compound; and b) determining whether the test compound alters the binding of the HB-954 ligand to the HB-954 polypeptide.

5. The method of claim 4, said method further comprising the steps of: c) adding a compound identified that alters binding of the HB-954 ligand to the HB-954 polypeptide in step (b) to an assay for the modulation of angiogenesis; d) determining whether the compound modulates angiogenesis; and e) identifying a compound that modulates angiogenesis in step (d) as a compound useful for the treatment of angiogenesis related diseases.

6. The method of any of claims 1 to 5, wherein the HB-954 polypeptide is expressed on the surface of a recombinant cell.

7. The method of claim 6, wherein said recombinant cell is an eukaryotic cell.

8. A method for identifying a compound useful for modulating angiogenesis, the method comprising: a) contacting a test compound with a cell expressing a HB-954 polypeptide; and b) determining whether the test compound alters activity of the HB-954 polypeptide in said cell;

9. The method of claim 8, said method further comprising the steps of: c) adding a compound identified that alters the activity of the HB-954 polypeptide in step (b) to an assay for the modulation of angiogenesis; d) determining whether the compound modulates angiogenesis; and e) identifying a compound that modulates angiogenesis in step (d) as a compound useful for the treatment of angiogenesis related diseases.

10. The method of claim 1, 4 or 8, wherein modulation of angiogenesis is inhibition of angiogenesis.

11. The method of claim 1, 4 or 8, wherein modulation of angiogenesis is activation of angiogenesis.

12. The method of claim 8 or 9, wherein the activity of the HB-954 polypeptide is determined by measuring the level of cAMP in the cell.

13. The method of claim 8 or 9, wherein the activity of the HB-954 polypeptide is determined by measuring the level of cytoplasmic Ca^{2+} in the cell.

14. The method of claim 8 or 9 wherein which the cell further contains a reporter gene operatively associated with a cAMP responsive element, and the level of cAMP is measured by measuring expression of the reporter gene.

15. The method of claim 8 or 9 wherein the activity of the HB-954 polypeptide is measured by measuring intracellular inositol 1,4,5- trisphophate (IP_3).

16. The method of claim 8 or 9 wherein the activity of HB-954 is measured by measuring intracellular 1,2-diacylglycerol (DAG).

17. A pharmaceutical formulation for the modulation of angiogenesis, comprising a compound that modulates the activity of HB-954, mixed with a pharmaceutically acceptable carrier.

18. A package comprising the pharmaceutical formulation of claim 17 and instructions for administering the pharmaceutical formulation for the purpose of modulating angiogenesis.

19. A gene therapy vector comprising a nucleic acid molecule that encodes HB-954 protein or a biologically active fragment thereof.

20. A nucleic acid molecule that is complementary to a nucleic acid molecule that encodes HB-954 protein or a fragment thereof.

21. A gene therapy vector comprising the nucleic acid molecule of claim 20.

22. The use of HB-954 protein or a biologically active fragment thereof in medicine.

23. The use of a monoclonal antibody which specifically binds an epitope of HB-954 protein or a biologically active fragment thereof in medicine.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/09619

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/705 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, BIOSIS, EMBASE, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 64872 A (LORA JOSE ;WHITE DAVID (US); MILLENNIUM PHARM INC (US)) 7 September 2001 (2001-09-07) page 46, line 20-24; claims 26,27,30; figure 15; examples 5,6	1-16, 19-23
X	HATA S ET AL: "CDNA CLONING OF A PUTATIVE G PROTEIN-COUPLED RECEPTOR FROM BRAIN" BIOCHIMICA ET BIOPHYSICA ACTA, AMSTERDAM, NL, vol. 1261, 1995, pages 121-125, XP000914574 ISSN: 0006-3002 figure 2	19-21

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 December 2003	19/12/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lanzrein, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/09619

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	ISHIZAKA NOBUKAZU ET AL: "Molecular cloning of a novel putative G protein-coupled receptor from rat aortic smooth muscle. Downregulation of the mRNA level by the cyclic AMP messenger pathway" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1218, no. 2, 1994, pages 173-180, XP009022214 ISSN: 0006-3002	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 03/09619

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 22, 23 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 17, 18
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 17, 18

Claims 17, 18 refer to modulators of HB-954 activity without giving a true technical characterization. No such compounds are disclosed in the application as filed and not even the natural ligand of HB-954 is disclosed. As a consequence, the scope of said claims is unclear and the subject-matter is not sufficiently disclosed and supported (Art. 5/6 PCT). Therefore, no meaningful search can be carried out for these purely speculative claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 03/09619

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0164872 A	07-09-2001	AU 4177801 A	12-09-2001
		WO 0164872 A2	07-09-2001

PATENT COOPERATION TREATY

PCT

REC'D 30 JUN 2004
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

(Rationalised Report according to the Notice of the President of the EPO published in the OJ11/2001)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
4-32303A/GTI		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP03/09619	29/08/2003	30/08/2002
International Patent Classification (IPC) or national classification and IPC		
C07K14/705		
Applicant		
NOVARTIS AG et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 2 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consists of a total of _____ sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17/03/2004	24/06/2004
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	ISERT B Tel. (+49-89) 2399 2828

Europäisches Patentamt • European Patent Office • Office européen des brevets

I. Basis of the report

The basis of this international preliminary examination is the application as originally filed.

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The question of whether the claimed invention appears to be novel, to involve an inventive step, or to be industrially applicable has not been the subject of the international preliminary examination in respect of the claims which have not been searched (Article 17(2)(a) or (3) and Rule 66.1(e) PCT); see also international search report).

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability

To the extent that the international preliminary examination has been carried out (see item III above), the following is pointed out:

In light of the documents cited in the international search report, it is considered that the invention as defined in at least some of the claims, which have been the subject of an international search report, does not appear to meet the criteria mentioned in Article 33(1) PCT, i.e. does not appear to be novel and/or to involve an inventive step (see international search report, in particular the documents cited X and/or Y and corresponding claim references).